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Review Article

Cytoplasmic lipid bodies in eosinophils: Central roles in eicosanoid generation

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Abstract

Lipid bodies are non-membrane bound, lipid-rich cytoplasmic inclusions that form in diverse cell types. Characteristically, lipid body numbers increase when eosinophils and other leukocytes are participating in inflammatory processes. Moreover, lipid bodies are numerous in other sites active in eicosanoid formation, such as the amnion epithelium at parturition. Our interests in lipid bodies relate to the roles that these structures play in arachidonate metabolism by eosinophils and other leukocytes involved in inflammation. Findings indicate that lipid bodies in these leukocytes can function as intracellular domains that are both depots of esterified arachidonate and sites at which regulated enzymatic events relevant to arachidonate metabolism can occur. Lipid bodies are discrete intracellular structures whose formation is specifically inducible early, whose increased numbers correlate with the 'priming' responses of leukocytes to form enhanced amounts of both cyclooxygenase- and lipoxygenase-derived eicosanoids and whose inhibition of formation correlates with reduced synthesis of these eicosanoids. Inhibition of lipid body formation represents a novel pharmacologic target to block the formation of eicosanoid mediators of inflammation.

Key words: aspirin, cyclooxygenase, eosinophils, leukotrienes, lipid bodies, lipoxygenase, platelet-activating factor.

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Introduction

Lipid bodies are non-membrane bound, lipid-rich cytoplasmic inclusions that form in diverse cell types.[1] While lipid inclusions exist in steroid-producing cells and pre-adipocytes and are storage sites of esterified cholesterol,[2] in most cells little is known about the origins, composition or functions of lipid bodies. In limited numbers, lipid bodies are normal constituents of many cells, including neutrophils (PMN), eosinophils, mast cells, macrophages, endothelial cells and fibroblasts.[1] Numbers of lipid bodies typically increase when cells are participating in varied inflammatory or pathologic processes.[1,3–7] In addition, lipid bodies are numerous in sites active in eicosanoid formation, such as the amnion epithelium at parturition.[8] Attention to lipid bodies is engendered by the roles that these cytoplasmic organelles play in arachidonate metabolism by eosinophils and other leukocytes involved in inflammation. Our findings indicate that lipid bodies function as intracellular domains that are both depots of esterified arachidonate and sites at which regulated enzymatic events relevant to arachidonate metabolism can occur. Lipid bodies constitute distinct structures whose formation is specifically inducible early (within 1 h), whose increased numbers correlate with the 'priming' responses of leukocytes to form enhanced quantities of both cyclooxygenase (COX)- and lipoxygenase (LO)-derived eicosanoids and whose inhibition of formation correlates with reduced synthesis of these eicosanoids. Inhibition of lipid body formation represents a new and feasible pharmacologic target to block the formation of eicosanoid mediators of inflammation.

We will review arachidonate metabolism pertinent to eicosanoid formation and current knowledge of lipid bodies, including their occurrence *in vivo*, their formation

in vitro and their constituents, and then consider the roles lipid bodies may play in the heightened elaboration of eicosanoids by cells involved in inflammation.

ARACHIDONIC ACID METABOLISM

Oxidative metabolism of arachidonate occurs by one of three pathways involving COX, LO or cytochrome P450 monooxygenase enzymes.[9,10] Products of these pathways, termed eicosanoids, have diverse biological activities.[9,11] Arachidonate and eicosanoids function as autocrine signals.[12] Eicosanoids are also potent paracrine mediators of inflammation.[13] The COX pathway, present in cells either as constitutive COX-1 and/or as an inducible COX-2 enzyme, forms prostaglandins (PG) and thromboxanes.[14–16] The 5-LO pathway, present in PMN, eosinophils, monocytes, mast cells and macrophages, forms 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotrienes (LT).[10] 15-Lipoxygenase, present in some leukocytes, especially eosinophils, is interleukin (IL)-4-inducible in other cells and active in inflammatory sites, including airways.[10] The formation of eicosanoids, not stored preformed within cells, is tightly regulated and enhanced formation of eicosanoids, necessary for their paracrine activities, occurs in response to activating stimuli.[10]

Eicosanoid formation is rate-limited in part by the availability of free arachidonate. Within cells, arachidonate is not free but is incorporated within glycerolipids,[17] especially phospholipids.[18] The availability of free arachidonate is governed by enzymatic reactions that cause its acylation[19] and its release from phospholipids. Little is known about arachidonate uptake into phospholipids and focus on initiation of arachidonate oxidative metabolism is directed towards enzymatic reactions that release it from phospholipids. An initial regulatory event involves activation of arachidonate-releasing phospholipases (PL). Increases in intracellular Ca^{2+} and phosphorylation activate some, but not all, PL. The importance of some PL and their ability to preferentially release eicosanoid-precursor arachidonate remain unsettled.[20] Specific cytosolic PLA_2 enzymes that are arachidonyl phospholipid-specific have been identified.[21,22] Specific cytosolic ($cPLA_2$) is an 85 kDa protein stimulated by low mmol Ca^{2+} and activatable by kinases, including mitogen-activated protein (MAP) kinases and protein kinase C (PKC).[22–24] The $cPLA_2$ undergoes Ca^{2+}-dependent translocation from the cytosol to 'membranes'.[25] Secretory PLA_2 also may contribute to release of eicosanoid-precursor arachidonate.[21] Thus, a critical regulatory step in eicosanoid synthesis is the regulation of PL activation, potentially involving MAP kinases and Ca^{2+}.

Once liberated, arachidonate becomes available for oxidative metabolism, although mechanisms coupling its release with subsequent utilization are not clear. Cyclooxygenase enzymes are associated with various cellular membranes, including the nuclear envelope and endoplasmic reticulum.[26] Based on the crystallographic structure of COX-1, COX are not transmembrane proteins but, rather, insert as monotopic proteins within one face of hydrophobic domains.[27] A second regulatory step in eicosanoid synthesis has been identified based on the induction by inflammatory stimuli of COX-2 expression.[16,28,29] The 5-, 12- and 15-LO enzymes are cytosolic, are activated by Ca^{2+} and may be dependent on other co-factors, including phospholipids.[30–32] Both 5- and 12-LO can undergo Ca^{2+}-dependent translocation from the cytosol.[30] For 5-LO, 5-LO-activating protein (FLAP) is necessary for cell expression of 5-LO activity, is a target for some 5-LO pathway inhibitors and apparently mediates translocation of cytosolic 5-LO enzyme.[33]

While translocation from the cytosol to membranes may facilitate interactions of cytosolic enzymes with arachidonate, there are increasing indications that compartmentalization of eicosanoid formation within cells may relate to the different autocrine or paracrine roles of eicosanoids. Thus, FLAP, 5-LO and COX can be found in the nuclear envelope,[26,34,35] a site proximate for intracrine signaling. In addition, COX-1 and COX-2, coupled to different stimulus-initiated pathways,[36] may use different endogenous arachidonate pools.[28] Moreover, there is increasing recognition that cells involved in inflammatory responses differ from usually studied normal cells in their pathways of arachidonate utilization.[7] At present, these differences are indicated by limited studies, but could help explain the capacities of eicosanoids to function separately as intracrine signals and paracrine mediators of inflammation.[10]

LIPID BODIES

In hypothesizing a role for lipid bodies in the generation of eicosanoid mediators of inflammation, several issues are critical in validating such potential functions for these cytoplasmic inclusions.

Lipid body occurrence *in vivo*

The ubiquity with which lipid bodies are found *in vivo* in cells associated with inflammation helps provide credibility for the hypothesized functions of lipid bodies. However, these lipid bodies are often missed on routine light microscopy (they are dissolved by desiccation and alcohol-based hematology stains[5]), conventional histopathology or immunofluorescent localizations (methanol or acetone fixation solubilizes lipid bodies[37]). With lipid fixation,[37] normal blood PMN and eosinophils contain approximately 1 and 5 lipid bodies/cell, respectively, whereas PMN from patients with infections and eosinophils from patients with eosinophilia contain many more lipid bodies/cell.[1,3,4,38] Induction of inflammatory exudates in animals with platelet-activating factor (PAF), specific antigen or casein[6,39] leads to increases in PMN lipid bodies. *In vivo*, increased lipid bodies in PMN associated with infectious, neoplastic and inflammatory reactions are found in tissues, blood and exudative effusions.[4,5,38] Even in the absence of inflammation, cells normally active in secreting PG, such as amniotic and renal epithelial cells,[8,40] contain increased numbers of lipid bodies. These *in vivo* findings with PMN, eosinophils and other cells[1,4,41,42] demonstrate that lipid body formation, a natural event in many cell types, is a morphologic correlate of the cells' participation in inflammation or generation of eicosanoids.

Sites of esterified arachidonate

Results from electron micrograph (EM) autoradiographic studies have demonstrated that [^{3}H]-arachidonate incorporated by human PMN,[4] eosinophils,[3,43] alveolar macrophages and mast cells, and murine and guinea pig peritoneal macrophages[41,44] was localized at lipid bodies. Parallel analyses of cellular lipids[3,4,41,44] and isolated eosinophil lipid bodies[43] demonstrated that incorporated [^{3}H]-arachidonate was principally esterified within phospholipid classes, establishing lipid bodies as sites of arachidonyl-phospholipid localization. Thus, lipid bodies function as sites of esterified arachidonate deposition in multiple types of leukocytes.

MECHANISMS OF LIPID BODY FORMATION

Although lipid bodies are prominent *in vivo* in cells associated with inflammatory reactions, it was unknown whether lipid body formation represented a physiologic response. We have demonstrated that lipid body formation in leukocytes is a highly regulated cellular response that develops rapidly (within 1 h), is elicitable by a restricted number of agonists and involves a cascade of activating steps. Leukocyte lipid body formation is induced by receptor-mediated PAF (but not other leukocyte agonists signaling through analogous G-protein-linked receptors) acting via the 5-LO pathway,[39] by *cis*-unsaturated fatty acids acting via an aspirin (ASA)/non-steroidal anti-inflammatory drug (NSAID)-inhibitable, but COX-1- and COX-2-independent, mechanism[38,44] or by diglycerides.[44] Both PKC and phospholipase C (PLC) activation[39,44] are involved in lipid body formation, which is dependent on tyrosine phosphorylation and *de novo* protein synthesis.[39] Moreover, lipopolysaccharide and hypoxia, stimuli that lead to heightened PG formation,[45,46] elicit lipid body formation.[47,48] These findings demonstrate that lipid body formation represents a deliberate, highly regulated and coordinated early response that generates cytoplasmic inclusions likely to be sites for eicosanoid synthesis.

PATHWAYS FOR INDUCTION OF LIPID BODY FORMATION

Platelet-activating factor-induced lipid body formation via a 5-lipoxygenase-dependent pathway

Receptor-mediated induction

Platelet-activating factor stimulated eosinophils and PMN lipid body formation that was dose-dependent (10^{-8} to 10^{-6} mol/L), developed rapidly within 15 min and increased over 1 h. Platelet-activating factor-induced PMN and eosinophil lipid bodies were ultrastructurally identical to native lipid bodies.[39] Platelet-activating factor signals via its seven transmembrane spanning receptor and several findings indicate that PAF induction of lipid body formation is receptor-mediated. First, lyso-PAF, an analog not binding to the PAF receptor, did not induce lipid bodies. Second, the PAF receptor antagonist WEB 2086 blocked PAF-induced lipid body formation. Third, G-protein-linked PAF receptor signaling is pertussis toxin-sensitive and pertussis toxin inhibited PAF-induced lipid body formation. Notably, in contrast to PAF, other PMN agonists, including leukotriene (LT)B_4, IL-8, C5a and formylmethionylleucylphenylalanine (fMLP), each also acting via seven transmembrane spanning G-protein-

linked receptors, failed to elicit lipid body induction. Thus, PAF, via a receptor-initiated pathway, was one specific stimulus for PMN lipid body formation.[39]

5-Lipoxygenase dependence of PAF-induced lipid body formation

Platelet-activating factor-induced lipid body formation was inhibited by lipoxygenase inhibitors, including the FLAP inhibitor MK886,[33] the anti-5-LO agent zileuton, diethylcarbamazine (DEC) and nordihydroguiaretic acid (NDGA). Platelet-activating factor-induced lipid body formation was not inhibited by COX inhibitors. To confirm the 5-LO dependence of PAF-induced signaling for lipid body formation, we evaluated PAF induction of lipid bodies in PMN and macrophages from normal and 5-LO knock-out mice.[49] Interestingly, 5-LO knock-out mice are known to be resistant to the lethal effects of PAF.[49,50] In PMN and peritoneal macrophages from normal mice, PAF stimulated lipid body formation; however, in cells from 5-LO-deficient mice, PAF failed to stimulate lipid body formation.[39] Thus, a requisite role for 5-LO in PAF-initiated induction of lipid body formation was established both with pharmacologic inhibitors of 5-LO activity and with genetically deficient 5-LO leukocytes.

As 5-LO activity was obligately required to mediate PAF-induced signaling for lipid body formation, we evaluated the enzymatic products of 5-LO that act to stimulate lipid body formation.[39] Notably, LTB_4 was not active. In addition, an LTB_4 receptor antagonist (CP 105 696) did not block PAF-induced lipid body formation, confirming the lack of a role for both exogenous and endogenous LTB_4 in PAF-initiated lipid body formation. Instead, 5(S)-HETE was the 5-LO product active in lipid body induction. 5(S)-Hydroxyeicosatetraenoic acid induced dose- and time-dependent increases in PMN lipid body formation. 5(S)-Hydroxyeicosatetraenoic acid was active at lower concentrations (10^{-9} to 10^{-7} mol/L) than PAF and was more active than 5(R)-HETE and 5-oxo-ETE.[39] Moreover, 5(S)-HETE induced lipid bodies in cells from 5-LO knock-out mice, cells unable to generate their own PAF-induced 5-HETE due to the absence of 5-LO.[39]

As with PAF itself, 5(S)-HETE-induced lipid body formation was inhibited by pertussis toxin. 5-Hydroxyeicosatetraenoic acid has previously been shown to stimulate PMN by a stereospecific, G-protein-linked, pertussis toxin-inhibitable mechanism, suggestive of a specific 5-HETE receptor.[51] Thus, PAF-induced lipid body formation proceeds through a 5-LO-dependent signaling cascade that generates 5(S)-HETE as an intracrine activator. These findings document one specific receptor-initiated pathway involved in the rapid induction of lipid body formation.

cis-Fatty acid-induced lipid body formation via an NSAID-inhibitable, COX-independent pathway

Fatty acid induction

cis-Unsaturated fatty acids, oleic acid (OA) and arachidonic acid (AA), stimulated lipid body formation in a non-cytotoxic, dose-dependent (0–10 mol/L) manner.[4,44] Lipid bodies formed rapidly, developing within 15 min and increasing in number over 1–2 h. Ultrastructurally, *cis*-fatty acid-elicited lipid bodies were identical to native lipid bodies.[4,44] Furthermore, these *cis*-fatty acid-induced lipid bodies incorporated [^{3}H]-arachidonate and [^{3}H]-palmitate identically to native lipid bodies.[4,44]

Activities of *cis*-unsaturated fatty acids

The activities of fatty acids as lipid body inducers were structurally restricted.[44] Fully saturated fatty acids (e.g. palmitate, myristate) and methyl esters of oleate and arachidonate were inactive.[44] Unsaturated fatty acids with *cis* geometries in their double bonds were considerably more active than those with *trans* double bonds and activity as inducers of lipid body formation increased with an increasing number of double bonds in C18 and C20 fatty acids. Lipid body inducing activities of *cis*-fatty acids were not due to fatty acid-derived or -elicited oxidants.[44] The stereochemically restricted potency of fatty acids as lipid body inducers suggested that a specific mechanism was involved.

NSAID dependence of *cis*-fatty acid-induced lipid body formation

To determine whether eicosanoids function in lipid body formation elicited by *cis*-fatty acids, potential COX and LO inhibitors were evaluated as inhibitors of *cis*-fatty acid-induced lipid body formation. Aspirin inhibited lipid body formation induced by *cis*-fatty acids in both human PMN and in eosinophils, as did the structurally unrelated NSAID indomethacin and piroxicam. Moreover, sodium salicylate (which does not substantially inhibit COX) was

as potent as ASA in inhibiting lipid body formation elicited by *cis*-fatty acids, suggesting that NSAID inhibition of lipid body formation was independent of COX inhibition. Neither ASA, indomethacin nor sodium salicylate inhibited [^{14}C]-AA uptake by human PMN stimulated by AA, indicating that these agents were not acting by diminishing fatty acid uptake. The lipoxygenase inhibitors zileuton, NDGA, DEC and MK886, did not inhibit *cis*-fatty acid-induced lipid body formation, in contrast to their inhibition of PAF-induced lipid body formation. Thus, ASA and varied NSAID, but not LO inhibitors, inhibited *cis*-fatty acid-induced lipid body formation.[38]

We next evaluated whether the inhibitory actions of ASA and NSAID were attributable to COX inhibition and several findings demonstrated that inhibition of *cis*-fatty acid induced lipid body formation by these agents was not COX-dependent.[38] First, the inhibition of lipid body induction by sodium salicyclate, an anti-inflammatory agent of uncertain action as it is unable to acetylate and inhibit COX enzymes, suggested a lack of dependence on COX inhibition. Second, *cis*-fatty acids, AA and OA all induced lipid body formation in macrophages from COX-1 and COX-2 genetically deficient mice identically as in macrophages from wild-type mice. Third, NSAID (indomethacin, sodium salicylate and NS-398) inhibited lipid body formation stimulated by both OA and AA in macrophages from COX-1 and COX-2 mice. Interestingly, NS-398, a nominally specific COX-2 inhibitor,[52] blocked lipid body formation in macrophages from wild-type and COX-1- and even COX-2-deficient mice, indicating that the anti-inflammatory actions of NS-398 are not restricted to COX-2 inhibition, but may also be based on inhibiting lipid body formation.

Thus, ASA and NSAID, via a COX-independent mechanism, inhibit *cis*-fatty acid-induced lipid body formation and this finding, together with related data on the suppression of eicosanoid formation presented later, expands the range of anti-inflammatory actions of these agents.[38]

1-Oleyl-2-acetylglycerol-induced pathway of lipid body formation

Diglycerides prime PMN for enhanced eicosanoid production.[53] We have demonstrated that the cell-permeable diglyceride, 1-oleyl-2-acetylglycerol (OAG), stimulated dose- and time-dependent lipid body formation in PMN.[44] 1-Oleyl-2-acetylglycerol-induced PMN lipid body formation was not inhibited by LO inhibitors or by NSAID. Hence, the rapid induction of lipid body formation in leukocytes by OAG did not involve either of the initial signaling pathways used by PAF (5-LO-dependent) or *cis*-fatty acids (ASA/NSAID-inhibitable).

INTRACELLULAR SIGNALING FOR LIPID BODY FORMATION

Having identified three specific classes of agonists and their initial pathways for initiation of lipid body induction, we further evaluated downstream intracellular signaling pathways involved in lipid body formation, including PKC and PLC.

Protein kinase C mediation

1-Oleyl-2-acetylglycerol and *cis*-fatty acids activate PKC in PMN and other cells and may differentially activate PKC isozymes.[54] That the activities of OAG and *cis*-fatty acids as well as PAF in inducing PMN lipid body formation were mediated in part by PKC activation was demonstrated with both PKC agonists and inhibitors. Other PKC activators, two PKC activating phorbol esters (phorbol myristate acetate (PMA), but not an inactive phorbol, stimulated lipid body formation.[44] Furthermore, PKC inhibitors 1-hexadecyl-2-O-methyl-glycerol, H-7, staurosporine and chelerythrine, blocked PMN lipid body formation elicited by diglyceride (OAG) and phorbol esters,[44] by *cis*-fatty acids (AA and OA)[44] and by PAF and 5-HETE.[39] Protein kinase C induces many changes in lipid metabolism in PMN (e.g. increases in diglycerides, phospholipid turnover and synthesis).[55,56] Our findings indicate that PKC-induced alterations in lipid metabolism occur in concert with what has been overlooked in biochemical studies, namely specific cellular responses that coordinately organize mobilized lipids and their associated proteins within the discrete, structurally defined domains of lipid bodies. Thus, PKC is centrally involved in lipid body formation elicitable with each of the three classes of lipid body inducers.

Phospholipase C mediation

In addition to PKC, PLC activation is involved in signaling lipid body formation, as evidenced by the actions of the two PLC inhibitors D609 and U 73122. These PLC inhibitors suppressed lipid body formation induced by *cis*-fatty acids, PAF, OAG and 5-HETE.[39] With PMN, combinations of PKC and PLC inhibitors have been cytotoxic and we have not been able to determine

whether blockade of both pathways is sufficient to completely inhibit elicited lipid body formation. As neither PKC nor PLC inhibitors completely suppressed induced lipid body formation, it is likely that PKC and PLC mediate parallel pathways that signal events involved in lipid body formation.

INTRACELLULAR MECHANISMS FOR LIPID BODY FORMATION

In addition to the signaling mechanisms noted earlier that are involved in lipid body formation, other fundamental biochemical processes are involved.

Tyrosine kinase activation

Tyrosine kinases (TK) are involved in the activation of arachidonate mobilization and eicosanoid formation.[57] Agents that inhibit TK, namely genistein and herbimycin A, inhibit the induction of lipid body formation elicited by PAF,[39] *cis*-fatty acids and OAG.

De novo protein synthesis

To evaluate whether new transcriptional and translational activity is required during the induction of lipid body formation in PMN, inhibitors of these steps were evaluated. Two inhibitors of translation, cycloheximide and puromycin, and a transcription inhibitor, actinomycin D, inhibited OAG-induced lipid body formation in PMN. Similarly, cycloheximide and actinomycin D inhibited lipid body induction stimulated by both PAF[39] and *cis*-fatty acids. These findings indicate that the stimulated induction of lipid body formation depends on new mRNA and protein synthesis. Thus, there must be activation of early response genes involved in the formation of lipid bodies and lipid bodies represent early response structures in cells.

SUMMARY OF DEFINED SIGNALING PATHWAYS FOR LIPID BODY FORMATION

In defining the mechanisms of lipid body induction in leukocytes, we have identified three classes of signaling agents and have helped determine the intracellular signaling pathways used by them. As shown schematically in Fig. 1, one pathway is initiated by receptor-mediated PAF signaling via 5-LO activation to generate 5-HETE, which then signals via a likely receptor-dependent mechanism. The second pathway initiated by *cis*-fatty acids is ASA/NSAID-inhibitable, but COX-independent. Diglyceride (OAG) constitutes a third defined initiating signal. For all three pathways, common downstream signaling requires PKC and PLC activation, TK activity and new mRNA and protein synthesis. Thus, the formation of lipid bodies arises from a highly coordinated series of sequential signaling and activating responses in leukocytes.

LIPID BODY FORMATION *IN VIVO*

To complement the *in vitro* studies that delineated pathways for lipid body formation, additional findings documented that lipid body formation accompanies inflammatory reactions *in vivo*. As PAF stimulates lipid body formation in PMN via a 5-LO-dependent signaling cascade, we evaluated whether administration of PAF *in vivo* would similarly generate leukocyte lipid bodies and whether this would be 5-LO-dependent.[39] Platelet-activating factor (1 mg) was administered into the pleural space of mice. Instillation of PAF resulted in little change in numbers of resident macrophages after 1 h in either wild-type or 5-LO knockout mice but, as expected, dramatically increased pleural cavity PMN from approximately 10^4 to 7×10^5/cavity in wild-type mice. In 5-LO knockout mice, the PMN influx after PAF was virtually absent. Intrapleural PAF significantly increased lipid body numbers in resident pleural macrophages and in recruited PMN. Moreover, this *in vivo* induction of lipid bodies in response to PAF was absent in pleural macrophages and in PMN from 5-LO-deficient mice. Thus, PAF stimulates lipid body formation via a 5-LO-dependent sequence *in vivo* as well as *in vitro*.[39]

ENZYMATIC MOBILIZATION OF ARACHIDONATE FROM LIPID BODIES

If lipid bodies constitute reservoirs of esterified arachidonate, then that archidonate must be liberated by specific PL. The freed arachidonate must either e translocated to sites where eicosanoid-forming enzymes act or these eicosanoid-forming enzymes must reside at or be translocated to lipid bodies. As the localization of arachidonate-mobilizing and -metabolizing enzymes at lipid bodies would provide critical novel information indicative of roles of lipid bodies in eicosanoid formation, we have actively sought evidence that these enzymes are present at lipid bodies. We have now established that all major proteins required for

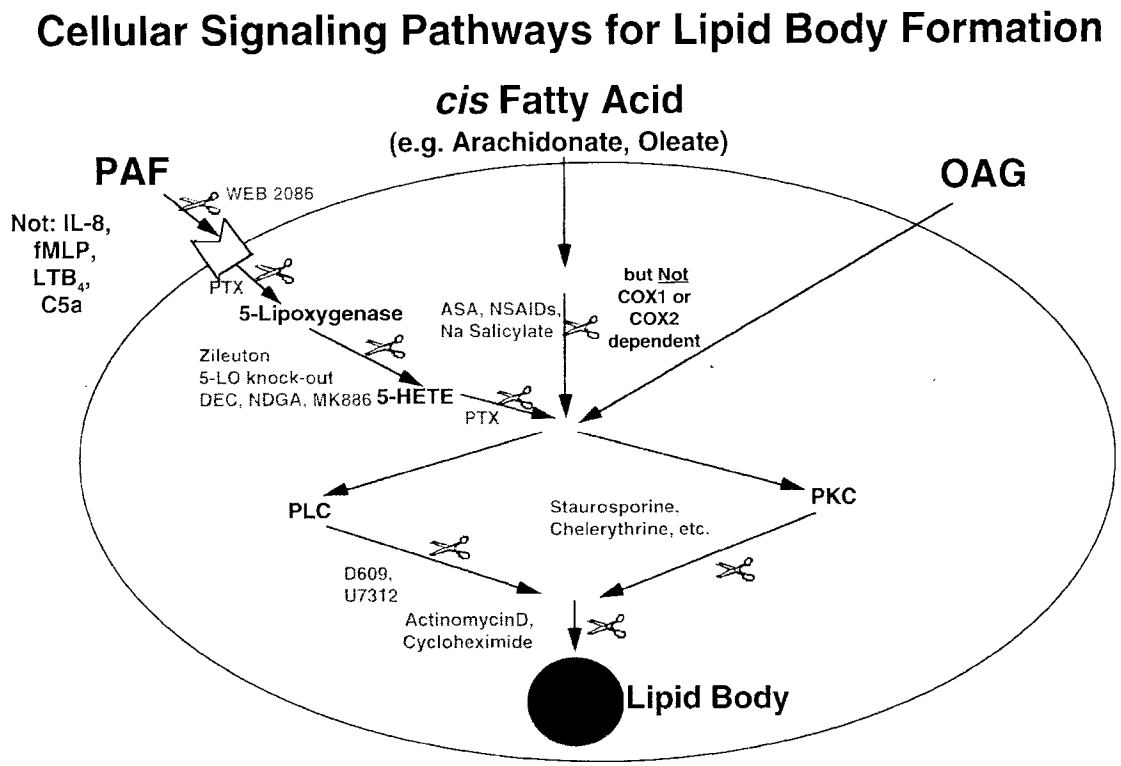

Fig. 1 Cellular signaling pathways for lipid body formation.

eicosanoid formation, including COX and LO themselves, are localized to lipid bodies.

SITES OF EICOSANOID-FORMING ENZYME LOCALIZATION

We have localized COX to lipid bodies in several cell types, including 3T3 fibroblasts (PF Weller *et al.*, unpubl. obs., 1996). Fibroblasts treated with rabbit anti-COX antisera, biotinylated second antibody and avidin–biotin complexed (ABC)-glucose oxidase or -alkaline phosphatase demonstrated diffuse perinuclear and endoplasmic reticulum anti-COX staining, fully consistent with that noted previously,[26,58,59] as well as distinct punctate anti-COX staining of lipid bodies (PF Weller *et al.*, unpubl. obs., 1996).

We corroborated lipid body localization of COX with immunogold EM and demonstrated that a variety of cells obtained from human airways by bronchoalveolar lavage contain COX in their lipid bodies.[60] Lipid bodies in alveolar macrophages incorporated [^{3}H]-AA and exhibited immunogold staining with an anti-COX monoclonal antibody (mAb) but not a control-irrelevant mAb.[60] With similar techniques, we localized COX by EM immunogold to lipid bodies in human eosinophils,[61] human lung mast cells, alveolar macrophages, type II pneumocytes and neutrophils,[60,62] murine 3T3 fibroblasts[61] and guinea pig peritoneal macrophages and line 10 hepatocarcinoma cells.[63] In these studies, control, negative staining with irrelevant mAb or with affinity-absorbed anti-COX mAb confirmed the specificity of immunogold localization of COX to lipid bodies. Thus, our ultrastructural studies, which previously localized [^{3}H]-AA to lipid bodies by EM autoradiography in a diversity of cells[3,4,41,43,44,62] now provide supporting data that COX is present in lipid bodies.

With similar approaches, we can localize other arachidonate-releasing and LT-synthesizing enzymes to leukocyte lipid bodies (PF Weller *et al.*, unpubl. obs., 1997). These novel findings support the capacity of lipid bodies to serve as sites for local eicosanoid formation. The co-compartmentalization of arachidonyl– phospholipids, $cPLA_2$, its activating kinases and eicosanoid-forming enzymes provides in one site an efficient means to regulate arachidonate release and couple it directly with oxidative metabolism to form eicosanoids.

USE OF LIPID BODY-DERIVED ARACHIDONATE FOR EICOSANOID SYNTHESIS

We have correlated lipid body induction with enhanced, primed synthesis of eicosanoids. Our hypothesis predicts that induction of lipid bodies will lead to enhanced formation of both COX- and LO-derived eicosanoids. We have extensively tested this prediction. All lipid body inducing stimuli lead to enhanced eicosanoid generation. Moreover, increasing lipid body numbers correlate directly with increasing levels of LO- and COX-derived eicosanoids generated by leukocytes. Human eosinophils or PMN were pre-incubated for 1 h with concentrations of distinct stimuli to induce lipid body formation: OA (a *cis*-fatty acid not an eicosanoid substrate), AA, OAG or PAF. Lipid bodies were enumerated, and replicate leukocytes were challenged with submaximal (0.25–0.5 mol/L) calcium ionophore A23187. Increasing concentrations of each of the four stimuli led to increasing lipid body numbers and, correspondingly, increasing LTC_4 production.[38,39] The increasing amounts of LTC_4 formed paralleled and statistically correlated with numbers of lipid bodies induced with each of the four stimuli.

Analogously, after leukocyte lipid bodies were induced with *cis*-fatty acids, magnitudes of LTB_4 and PGE_2 production correlated with PMN lipid body numbers and amounts of LTC_4 correlated with eosinophil lipid body numbers.[38] Arachidonic acid, as well as OA, dose-dependently induced concordant increases in both lipid body numbers and priming for enhanced LTB_4, PGE_2 and LTC_4 production. Increased numbers of AA- and OA-induced lipid bodies in both cell types correlated with the increased quantities of each eicosanoid generated by the primed leukocytes. Notably, stimulation of leukocytes with OA, not itself an eicosanoid precursor fatty acid, led to increments in the production of each eicosanoid that quantitatively paralleled the incremental increases in lipid body numbers (PGE_2 $r = 0.99$, $P < 0.01$; LTB_4 $r = 0.99$, $P < 0.01$; LTC_4 $r = 0.95$, $P < 0.05$). Thus, the capacity of two different leukocyte types to generate greater quantities of COX and LO pathway derived eicosanoids correlated with levels of lipid bodies in these cells.[38]

With PAF as a stimulus we have evaluated both the dose and time dependence of lipid body induction and priming for enhanced eicosanoid release in human PMN.[39] The PMN were incubated with increasing concentrations of PAF (10^{-8} to 10^{-6} mol/L) for 5 min or 1 h. After incubation, lipid bodies were enumerated and replicate leukocytes were stimulated with submaximal A23187 (0.5 μmol/L). Platelet-activating factor induced dose-dependent increases in PMN lipid body numbers after 1 h, but not as early as 5 min after stimulation. Incubation with PAF for only 5 min failed to prime PMN for enhanced LTB_4 or PGE_2 production. However, after 1 h of PAF stimulation, significant dose-dependent priming for enhanced production of both LTB_4 and PGE_2 was noted. Platelet-activating factor induced 5.5- and 2.8-fold increases in the production of LTB_4 and PGE_2, respectively, at the highest PAF concentration (10^{-6} mol/L). Furthermore, there were statistically significant, positive correlations between the PMN content of lipid bodies and priming for enhanced LTB_4 ($r = 0.986$, $P < 0.001$) and PGE_2 ($r = 0.971$, $P < 0.001$) generation.[39] Thus, there were both dose- and time-dependent correlations between lipid body formation and enhanced eicosanoid formation, further indicating that lipid bodies contribute to eicosanoid formation.

In all of these studies with each stimulus for lipid body induction, the quantities of produced eicosanoids, both COX- and LO-derived, demonstrated statistically significant correlations with the numbers of induced lipid bodies. While this is correlative and not alone definitive proof that lipid bodies are sites of augmented eicosanoid synthesis, these findings are fully supportive of potential roles for lipid bodies in enhanced eicosanoid production.

As the induction of COX-2 by inflammatory stimuli represents one inducible mechanism to augment prostanoid production, we determined which COX was responsible for enhanced PG production by evaluating the effects of COX-1 or COX-2 deficiency on priming.[38] Primed COX-2-deficient macrophages were found to be indistinguishable from wild-type cells in both lipid body and PGE_2 formation, indicating that COX-2 does not have a role in enhanced PG production. However, primed COX-1-deficient macrophages demonstrated severely diminished levels of PGE_2, identifying COX-1 as the enzyme responsible for enhanced PGE_2 production induced early in AA-primed macrophages. As the induction of COX-2 expression by stimuli including LPS[57] requires up to 48 h, our findings with lipid bodies identify lipid body induction as a more rapid and COX-2-independent means to increase production of both COX- and LO-derived eicosanoids.

CONCOMITANT INHIBITION OF LIPID BODY INDUCTION AND EICOSANOID FORMATION

If the hypothesis that lipid bodies have roles in augmented eicosanoid formation is valid, then the

inhibition of lipid body formation should result in suppressed eicosanoid formation. We have tested this prediction and have validated it with several types of inhibitors.

Aspirin inhibition of induction of enhanced eicosanoid generation

We evaluated whether aspirin (ASA) inhibition of lipid body formation would affect *cis*-fatty acid-induced priming for enhanced production of both COX- and lipoxygenase-derived eicosanoids.[38] Aspirin pretreatment inhibited PGE_2 production by human PMN and eosinophils in vehicle- and *cis*-fatty acid-stimulated leukocytes, but this ASA inhibition of prostanoid production may be due solely to ASA inhibition of COX. In contrast, ASA is devoid of direct inhibitory effects on LO in leukocytes and platelets.[64–66] We have demonstrated that ASA significantly inhibits AA- and OA-induced priming for enhanced production of 5-LO-derived LTB_4 by PMN and LTC_4 by eosinophils and that sodium salicylate similarly inhibited *cis*-fatty acid-induced priming for enhanced LTB_4 production by PMN. Aspirin was not acting to block pathways of AA mobilization or metabolism activated by calcium ionophore, as aspirin failed to inhibit calcium ionophore-induced LTB_4 and LTC_4 production in cells not prestimulated with *cis*-fatty acids and was not inhibiting eicosanoid export (no residual intracellular eicosanoids were detectable). The inhibition by aspirin of priming for enhanced eicosanoid production correlated with its ability to inhibit *cis*-fatty acid-induced lipid body formation.[38]

CYCLOHEXIMIDE–ACTINOMYCIN D INHIBITION OF ENHANCED EICOSANOID GENERATION

Because the protein synthesis inhibitors actinomycin D and cycloheximide were effective in blocking lipid body formation induced by PAF and lipid body numbers correlated with enhanced eicosanoid formation, we examined whether protein synthesis inhibitors would inhibit PAF-induced priming for eicosanoid production.[39] Pretreatment of PMN with actinomycin D (1 μmol/L) or cycloheximide (1 μmol/L) inhibited not only PAF-induced lipid body formation, but also priming for enhanced LTB_4 and PGE_2 production.[39] Protein synthesis inhibitors were not acting to block pathways of arachidonate release or metabolism that are activated by calcium ionophore, as actinomycin D and cycloheximide failed to inhibit calcium ionophore-induced LTB_4 and PGE_2 production in cells not prestimulated with PAF.[39]

Thus, inhibitors of lipid body induction, whether they be ASA/NSAID blocking OA- or AA-induced lipid body formation or inhibitors of mRNA and protein synthesis, also inhibited the enhanced production of both COX- and LO-derived eicosanoids. This provides additional support for the hypothesized roles of lipid bodies as inducible sites active in the enhanced generation of eicosanoid mediators of inflammation. Moreover, the capacity of inhibitors of lipid body induction to block eicosanoid formation identifies a novel target for anti-inflammatory drug action. Notably, the suppression by ASA and NSAID of lipid body production and of LT and PG production, by a COX-independent mechanism, expands our understanding of the anti-inflammatory actions of these drugs. Aspirin and NSAID block *cis*-fatty acid-induced lipid body formation and concomitantly inhibit formation of both COX- and LO-derived eicosanoids.

RELEVANCE OF LIPID BODIES TO EICOSANOID PRODUCTION BY INFLAMMATORY CELLS

If lipid bodies have specific roles in contributing to eicosanoid mediator formation in inflammation, lipid bodies may provide distinct advantages.

As membrane-sparing domains

Eicosanoids derive from arachidonyl-phospholipids assumed to reside in membranes, but which membranes and, indeed, whether it is membranes that serve as depots of precursor arachidonate are unknown. In one study, cells lost their capacity to form PGE_2 after deprivation of exogenous arachidonate for 24–48 h.[67] As total cellular arachidonate and relevant enzymes remained normal, arachidonate depletion from an unidentified cellular pool was hypothesized.[67] Under the conditions used, lipid bodies would most likely have been depleted.[68] Another consideration concerning membrane structure is pertinent. Using lipid bodies, not membranes, as a source of eicosanoid-precursor arachidonate obviates excessive membrane perturbation when quantities of arachidonate are removed from membrane phospholipids. Hence, relatively small quantities of arachidonate may be liberated from membranes when that arachidonate forms autocrine eicosanoids, as elicited by signal-transducing mechanisms within membranes, whereas larger amounts of arachidonate needed for forming eicosanoid paracrine mediators could be derived from lipid body stores.

As a basis for leukocyte priming

In studies of PMN there has been an apparent paradox. In response to ionophore A23187, PMN generate large quantities of 5-HETE and LTB_4, whereas more natural, receptor-mediated stimulation (e.g. fMLP) routinely elicits little eicosanoid formation[53,69] unless PMN are first 'primed'. 'Priming' consists of pre-incubating PMN with OAG, PAF, arachidonate, PMA or lipopolysaccharide (LPS)[45,53,69] Of note from our studies, these same priming agents in the concentrations and time periods used, would induce abundant lipid body formation.[38,39,44] Analogously, pre-incubating cells with 10–50 mol/L arachidonate prior to stimulation (as done nominally to provide ample substrate) also stimulates lipid body formation.[38] Priming agents similarly augment eicosanoid formation by A23187-stimulated PMN.[38,39,70,71] Thus, the capacity of PMN to release eicosanoids in response to physiologic stimuli could be correlated with their content of lipid bodies and 'priming' for eicosanoid release could be based, at least in part, on lipid body induction.

We have documented a relationship between lipid body formation and primed responsiveness for augmented eicosanoid formation. With each class of lipid body inducer (PAF, OAG, *cis*-fatty acids, LPS), we have now established that numbers of induced lipid bodies correlate with magnitudes of enhanced COX- and LO-derived eicosanoids. Moreover, inhibition of lipid body induction with various inhibitors correlates with suppression of priming for enhanced eicosanoid generation. Aspirin inhibition of OA-induced lipid body formation even suppresses generation of 5-LO (not itself ASA-inhibitable)-derived LT. Thus, prior induction of lipid body formation is associated with the capacity for primed generation of eicosanoids, and inhibition of lipid body formation is associated with diminished capacity to form both COX- and LO-derived eicosanoids.

As an inducible early mechanism to enhance inflammation

In contrast to normal leukocytes studied *ex vivo*, *in vivo* it is cells associated with inflammation, those likely to contain many lipid bodies, that are producing quantities of eicosanoids.[7] For instance, PMN from casein-elicited rodent peritoneal exudates produced more LTB_4 than did normal PMN[72] and activated 'hypodense' human eosinophils (which contain many lipid bodies[3]) form more LTC_4 than normal eosinophils.[73] In leukocytes and other cells associated with inflammation, the mobilization of arachidonate for eicosanoid formation may differ from that studied in more readily obtainable normal blood leukocytes.[7] The capacity of leukocytes to rapidly form lipid bodies in association with inflammatory processes provides a means by which these cells can rapidly develop specialized intracellular domains that augment their capability to generate eicosanoid mediators of inflammation. In comparison with the induction of COX-2, which occurs over 2–48 h and augments only PG production, induction of lipid body formation develops within 1 h and is associated with enhanced formation of both PG and LT. Thus, the induction of lipid body formation is a distinct, early developing response that constitutes a means to augment the generation of diverse eicosanoid mediators of inflammation.

As a site for cytokine actions

A novel finding that adds yet another potential dimension to the functions of lipid bodies is the recognition that lipid bodies are sites of localization of some cytokines, notably tumor necrosis factor (TNF)-α.[74] Our initial indications that lipid bodies may be sites of localization of the cytokine TNF-α arose during studies of human eosinophils,[75] in which immunolocalization at the light microscopic level demonstrated lipid body staining with anti-TNF-α mAb. To confirm this finding and to evaluate its pertinence *in vivo* we used ultrastructural immunogold localization of TNF-α to evaluate infiltrating inflammatory leukocytes in colonic lesions of Crohn's disease.[74] Eosinophils, mast cells, neutrophils, macrophages, epithelial cells and other leukocytes exhibited specific immunogold staining for TNF-α in sites uniformly included lipid bodies.[74] Controls, including irrelevant mAb and anti-TNF-α mAb immunoabsorbed with solid-phase TNF-α, were negative.[74] While TNF-α was also located in other sites within these cells, in each of the leukocytes present in sites of Crohn's colitis, lipid body domains exhibited specific immunogold localization for TNF-α.[74] The lipid body localization of TNF-α, initially recognized as cachectin due to its lipolytic activities, is intriguing because of the role that TNF-α may play in eicosanoid metabolism.

SUMMARY

Lipid bodies represent specialized intracellular domains. Their prominence *in vivo* in natural and experimental

inflammation supports their relevance to natural inflammation. In addition, the specificities of the cellular signaling pathways that rapidly induce lipid body formation indicate that their formation is a deliberate and well-regulated process. The dependence of lipid body induction on *de novo* mRNA and protein synthesis makes lipid bodies structural analogs and, probably, in part products of early response genes. These structures contain esterified eicosanoid-precursor arachidonate. An arachidonate-releasing phospholipase ($cPLA_2$) and its activating kinases (MAP kinases and PKC) are present and active at lipid bodies and eicosanoid-forming enzymes (COX and 5- and 15-LO) localize to lipid bodies. Lipid body numbers correlate with priming for enhanced eicosanoid synthesis and inhibition of lipid body formation is associated with loss of this priming. Thus, the heightened presence of lipid bodies in cells likely to be generating eicosanoids as part of inflammatory reactions is a reflection of the functions of lipid bodies as dynamic, rapidly inducible, specialized intracellular domains with roles in the metabolic transformation of arachidonate into paracrine mediators of inflammation.

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